

First report of azole-resistant *Aspergillus* species in poultry feed in Kabale, South West, Uganda

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Abstract

Widespread use of azoles in clinical and environmental settings has favored the selection of azole-resistant *Aspergillus* species necessitating antifungal susceptibility studies understanding their prevalence and resistance profiles in those settings. However, there is still limited information available on these strains in Africa. The objective of this study was to assess the environmental prevalence of Azole-resistance *Aspergillus* strains in poultry feed in Kabale, South West Uganda. The study evaluated the susceptibility profile of *Aspergillus* and other fungi isolates from 10 poultry feeds across 10 different commercial outlets. Isolates were first identified using rDNA 18S genomic sequencing via ITS 1 and 2 primer combination and confirmed using Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF). Seventeen fungal strains were identified of which *Aspergillus flavus* was the most prevalent. All *Aspergillus* and one *Penicillium* isolates were further screened for azole-resistance using azole-containing agar plates (itraconazole, voriconazole, posaconazole, isavuconazole) and fluconazole antifungal susceptibility strip method at 4 mg/L, which was the first line of treatment. *Aspergillus chevalieri*, *A. tamari*, *A. flavus*, *P. citrinum* fungi isolates showed resistance to fluconazole. The EUCAST susceptibility testing method did not show any of the isolates resistant to tested azoles (itraconazole, voriconazole, posaconazole, isavoriconazole) or amphotericin B. Overall, we found resistance to fluconazole. This study is the first investigative study of *Aspergillus* species in poultry feed in Kabale, South West, Uganda.

Keywords: azole resistance; environment; fungi; poultry

Introduction

Drug resistance in *Aspergillus* species is a public health risk that is growing globally. Antifungal drugs give treatments for infections in both humans and animals and the preferred class of antifungal agents for treating human aspergillosis is azoles. However, some of these antifungal drugs occasionally promote resistance and weaken human resistance to diseases. Antimicrobial resistance is a natural process by which pathogens

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(bacteria, viruses, parasites, fungi, and other microbes) become resistant to the medications used to treat them. Antimicrobial resistance (AMR) is a key global public health emergency now emphasized in the One Health Initiative Task Force (World Health Organization, 2022). Fungi have a significant role in developing diseases in both plant and animal species, including humans (Buckley, 2008). The lack of novel fungicides with distinct mechanisms of action furthers the issue of antifungal resistance selection in the environment (Oshero and Kontoyiannis, 2017).

The abuse and overuse of antimicrobials, self-medication, excessive antibiotic prescription to patients, high nosocomial infection rates, usage of antibiotics in fish and cattle husbandry, poor sanitary conditions for humans and animals, ineffective infection prevention and control strategies in the community, are just a few of the causes of AMR in developing countries, some of which are also applicable in high income countries. (UNAS, 2015; Mbonye *et al.*, 2016; Odoi and Joakim, 2021; WHO, 2021) However, there is insufficient data on *Aspergillus* triazole susceptibility in Africa and the resulting resistance in diseases that impact animal, human, and environmental health. It is therefore essential to evaluate the global movement of AMR emergence related to poultry production (FAO, 2014; Laxminarayan *et al.*, 2015; Van Boeckel *et al.*, 2019). With over 80% of the population reportedly working in agriculture, Uganda is a low-income country where agriculture is the backbone of the economy. The growth in food production globally can be attributed to a number of factors which include higher incomes and urban population expansion, the demand for high-quality sources of animal products will continue to rise. In order to meet the demands of society, and also for the economy, more agricultural and livestock goods must be produced (Schmidt, 2012). Africa has already seen a 64% increase in meat output since year 2000, primarily as a result of consumer choices, urbanization, and the need for high-protein diets (Van Boeckel, 2019; Otte *et al.*, 2019). As a result, chicken production techniques will keep transitioning from subsistence farming methods to an intensive food production system, which involves the frequent use of antimicrobials. Antibiotics are commonly accessible at neighborhood pharmacies, but there are growing worries about their unauthorized and unprescribed use throughout the nation (Ikwap *et al.*, 2014). With the use of available medications for both people and animals in Uganda, it has been speculated over the past several years that their unrestricted usage and availability are contributing to the rise of AMR and a decline in effective treatment outcomes (Tadesse *et al.*, 2017). Van Boeckel (2019) reported that the majority of antimicrobial use (AMU) is for animal food production. Nonetheless, Mshana *et al.* (2021) reported that the amount of antimicrobial use in Africa fell from 4279 tonnes in 2017 to 3558 tonnes in 2020. Of the total amount used, tetracycline was the most common and accounted for 63% in 2016, 11.6% in 2017, 31.7% in 2018, and 28.7% in 2020. Resendiz Sharpe *et al.* (2018) in their work also reported that due to the widespread use of antifungals in agriculture, the prevalence of antifungal-resistant fungal strains is rising. Therefore, there is an overall need to determine the impact AMU has into the diversity and type of resistant fungal strains present in poultry feed in developing countries.

Materials and Methods

Collection of samples

Five grams (5 g) of poultry feed (layers and starter mash) were collected from 10 different shops in the Kabale metropolis, South-West, Uganda (Figure 1). Samples were collected in sterile Ziploc bags and sealed immediately.

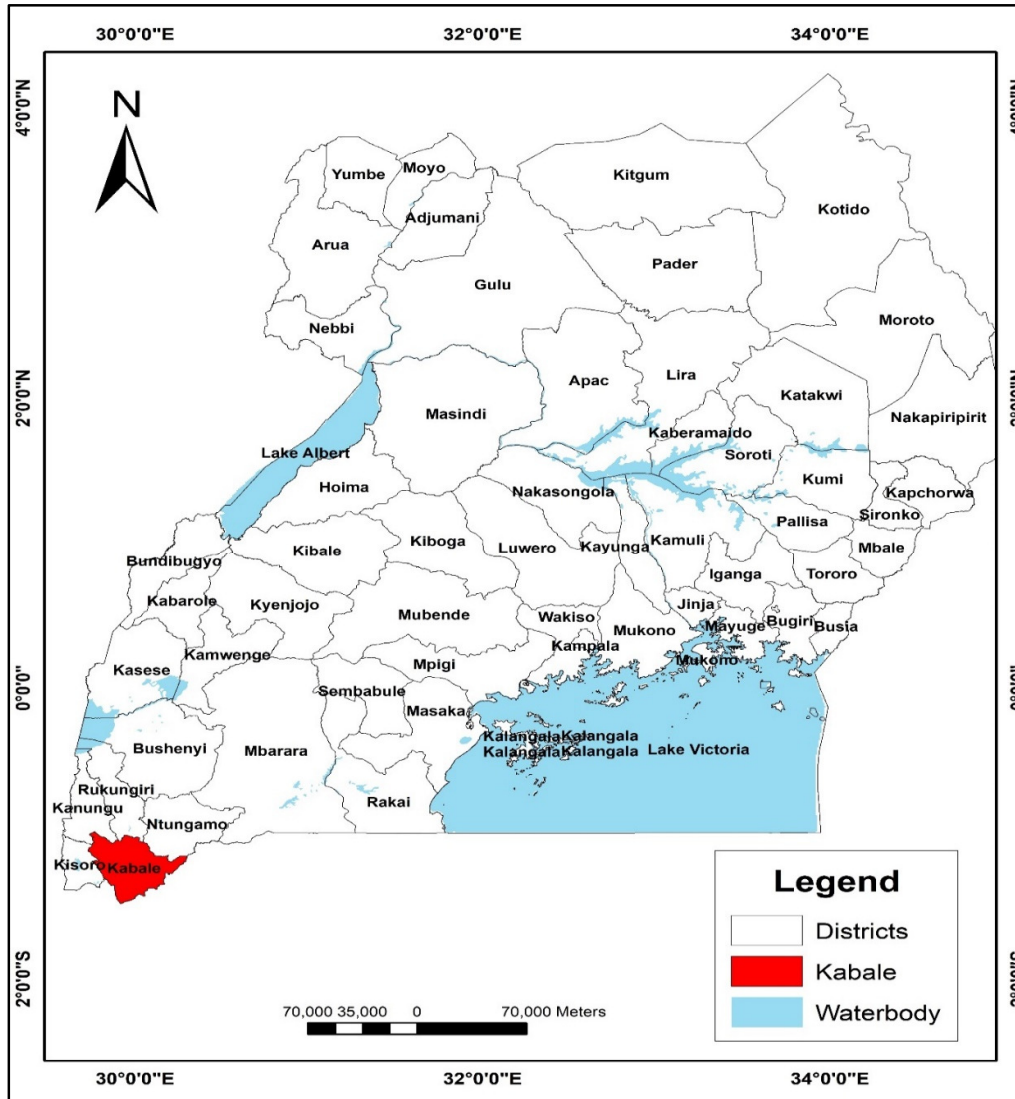


Figure 1. Map showing Kabale district

Media preparation

Sabouraud Dextrose Agar were all prepared and sterilized according to the manufacturer’s instructions, kept molten at 45 °C in a water bath and poured aseptically over serially diluted dispensed samples in sterile petri dishes (20 ml per dish). To prevent moisture from seeping back into the culture, petri dishes were gently mixed and incubated with the lid facing downward.

Serial dilution and sub culture methodology

The method adopted for serial dilution was that of Obuekwe and Ogbimi (1998). One gram of each sample was first measured and then dissolved in 10 mL of sterile distilled water before serial dilution. Next, a one milliliter aliquot was diluted with 9 mL of sterile water in different test tubes to give a 1:9 dilution. Finally, one milliliter of the sample was plated on SDA augmented with chloramphenicol, in a pour plate method, swirled on the work surface, and given time to settle. For five to seven days, the plates were incubated with their tops facing down. At room temperature, triplicates of each plate were incubated.

With the aid of an inoculating needle, fungal isolates on primary plates were subcultured and plated on freshly produced Sabouraud dextrose agar (Oxoid) supplemented with chloramphenicol to prevent growth of bacteria on properly labelled plates. Three to five days of incubation at room temperature was observed.

Molecular identification of fungi species

Species identification was carried out based on DNA barcode sequences. These barcode sequences are short DNA sequences internationally agreed upon as genetic markers. The most used genetic marker for fungi is the Internal Transcribed Spacer (ITS) region. Primers 1 and 2 were employed in this study to sequence the ITS region. After DNA extraction, polymerase chain reaction (PCR) was used to amplify copies of the partial internal transcribed spacer (ITS) fragment of rDNA (Odebode *et al.*, 2020). The success of the PCR product was assessed by undertaking gel electrophoresis on a 1% agarose gel in Tris-acetate EDTA buffer. After electrophoresis, DNA was visualized by SYBR-safe staining. Next, a PCR purification step was done to remove unutilized dNTPs, primers, polymerase, and other PCR compounds to get a well purified DNA template for sequencing. Next-generation sequencing was carried out at Eurofins Scientific Belgium using Illumina Bead Chip arrays. The obtained sequences were then compared to the National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) to identify the closest related species.

MALDI-TOF identification of fungal isolates

Identification of selected isolates was further confirmed using Matrix- assisted laser desorption ionization –time of flight mass spectrometry (MALDI-TOF) as previously described (Resendiz-Sharpe *et al.*, 2021). Briefly, fresh isolates (24 hrs) were gently scraped with a sterile scalpel and then transferred in a mixture of sterile water and anhydrous ethylic alcohol for protein precipitation. Samples were subsequently treated with 70% formic acid (5 min incubation) followed by 100% acetonitrile (5 min incubation) for protein exposure. Three duplicates of one micro liter of the supernatant extract from each isolate were placed on a section of the target plate, and when dry, one microliter of the matrix solution was added. The Bruker Biotyper MALDI-TOF MS system was used to determine the spectrum (Bruker Daltonics, Bremen, Germany) and identified using the mass spectrometry identification (MSI) platform (Normand *et al.*, 2017).

The same fungal isolates used for ITS sequencing were likewise used for MALDI-TOF identification.

Antifungal susceptibility test

Etest

The Etest was selected for four fungal isolates. Fungal mycelium was harvested from freshly sub cultured fungi isolates on Sabouraud dextrose agar enriched with chloramphenicol. Turbidity was adjusted to the 0.5 McFarland standard and one to two colonies were suspended in saline (NaCl 0.85%). Susceptibility testing was conducted using Petri dishes (150 mm) with RPMI-1640 medium supplemented with 2% glucose and pH adjusted to 7.0 using MOPS (0.165 M, pH 7.0) buffer. Fungal suspension was evenly applied to the medium's surface using a sterile cotton swab. The manufacturer (bioMérieux specified) test technique was followed. The Etest strips were applied after the Petri plates had dried for 10 to 15 minutes. When adequate growth was evident after 24-48 hours of incubation at 35 °C, minimum inhibitory concentration (MIC) values were taken. Minimum Inhibitory Concentration was determined using the EUCAST (European Committee on Antimicrobial Susceptibility Testing) reference method for filamentous fungi and clinical breakpoints of molds were used to confirm the azole-resistant phenotype of fungi isolates and epidemiological cut-off values for species was used where breakpoints have not been determined. Resistance to these antifungals often falls into areas of technical uncertainty.

Well agar plate test for Fluconazole screening

One agar well plate contained no azole antifungal (growth control) and two supplemented with fluconazole (4 mg/L). The plates were then incubated at 50 °C for 72-96 h. The growth control medium was used to determine resistance.

Triazole-resistance screening

Triazole resistance screening was done for five selected isolates using the commercially available 4-well agar plate dilution method VIPCheck® (Media Products, Groningen, The Netherlands), following the manufacturer's recommendation. In a nutshell, all isolated colonies that grew were swabbed to make a suspension (0.5 McFarland standard) in sterile water. Of this suspension, 25 µl were added to each well and incubated at 36 °C. Growth was recorded at 24 and 48 hours. Growth on any of the triazole agars prompted MIC determination (European Committee on Antimicrobial Susceptibility Testing, 2022).

EUCAST broth micro dilution method for filamentous fungi

Minimal inhibitory concentrations (MICs) of itraconazole, voriconazole, posaconazole and amphotericin B (all from Sigma-Aldrich, USA) for *Aspergillus flavus*, *A. ochraceus*, *A. niger*, *A. stellatus* and *Penicillium hetheringtonii* were determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth micro dilution reference method for filamentous fungi. Triazole-resistant phenotype was confirmed if at least one MIC value was above the established EUCAST resistance clinical breakpoints when available (e.g., *A. fumigatus* = voriconazole >1, itraconazole >1 mg/L, posaconazole >0.25, mg/L, and Amp B) (EUCAST, 2022)

Data analysis

Results were done using Microsoft Office Excel.

ResultsMolecular identification of fungi species

Seventeen fungal species were identified from the feed samples as shown in Table 1.

Table 1. List of fungi identified

Identified fungi	Species
K 01	<i>Penicillium citrinum</i>
K 02	<i>Aspergillus tamari</i>
K 03	<i>Aspergillus chevalieri</i>
K 04	<i>P. corylophilum</i>
K 05	<i>A. flavus</i>
K 06	<i>Hyphopichia burtonii</i>
K 07	<i>Meira argovae</i>
K 08	<i>Meria nashicola</i>
K 09	<i>A. niger</i>
K 10	<i>Neurospora crassa</i>
K 11	<i>P. hetheringtonii</i>
K 12	<i>P. steckii</i>
K 13	<i>Paraphyton mirabile</i>

K 14	<i>P. nilense</i>
K 15	<i>A. ochraceus</i>
K 16	<i>A. stellatus</i>
K 17	<i>Fusarium proliferatum</i>

Antifungal susceptibility testing by concentration Gradient strip Test method

E test strip containing fluconazole was used as antifungal resistance screening against selected fungi namely: *Aspergillus chevalieri*, *A. tamari*, *A. flavus*, *Penicillium citrinum*. After 24 hours, no visible growth was observed on the plates but after 48 hours there was fungi growth. E test strip plate's shows resistance to Fluconazole strip (Figure 2).

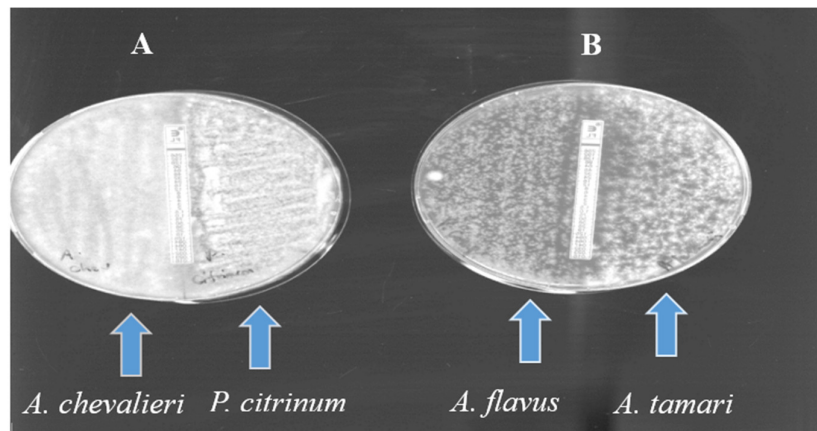


Figure 2. E test strip result of *Aspergillus chevalieri*, *Penicillium citrinum* (A) and *A. flavus*, *A. tamari* (B) fungi isolates grown at room temperature on SDA showing resistance to fluconazole

Fluconazole antifungal resistance result

Fluconazole was used as the first set of antifungal drugs against two selected fungi because of their abundance in all feed sampled (*A. chevalieri* and *A. tamari*). 6-well agar plates containing fluconazole antifungal drug (4 mg/L) were tested against selected isolates but resistance to the drug was observed in all the tested isolates. There was visible growth in all the wells (Figures 3 and 4).



Figure 3. 6 well agar plates containing fluconazole (4 mg/L) showing growth of *Aspergillus chevalieri* after 72 hours at room temperature

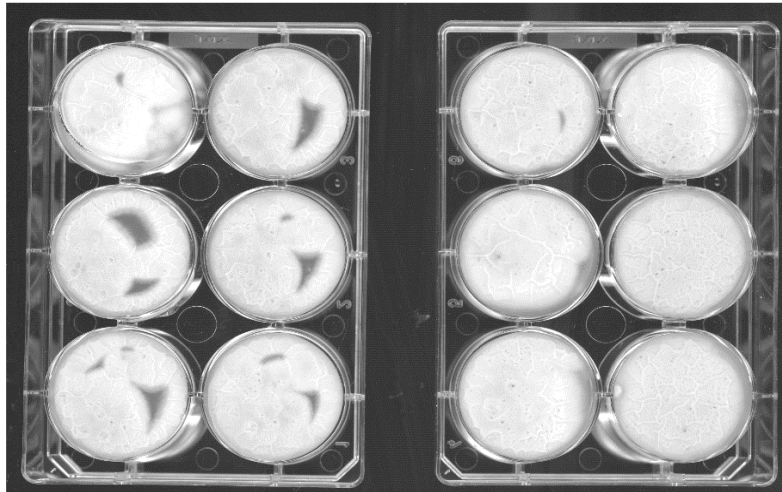


Figure 4. 6 well agar plates containing fluconazole (4 mg/L) showing growth of *A. tamari* after 72 hours

EUCAST susceptibility test

The MICs of voriconazole, isavuconazole, posaconazole, itraconazole and amphotericin B, as they are the antifungal medications used were determined as shown in Table 2. None of the isolates showed resistance to the antifungals.

Table 2. Anti-fungal (EUCAST) susceptibility test results

Isolates	Antifungal	MIC value
<i>Aspergillus flavus</i>	amphotericin B	2 mg/L
	voriconazole	1 mg/L
	isavuconazole	1 mg/L
	posaconazole	0.125 mg/L
	itraconazole	0.25 mg/L
<i>A. ochraceus</i>	amphotericin B	1 mg/L
	voriconazole	0.125 mg/L
	isavuconazole	0.125 mg/L
	posaconazole	0.125 mg/L
	itraconazole	0.25 mg/L
<i>Aspergillus niger</i>	amphotericin B	0.25 mg/L
	voriconazole	2 mg/L
	isavuconazole	2 mg/L
	posaconazole	0.25 mg/L
	itraconazole	0.40 mg/L
<i>Penicillium hetheringtonii</i>	amphotericin B	1 mg/L
	voriconazole	0.5 mg/L
	isavuconazole	0.5 mg/L
	posaconazole	0.125 mg/L
	itraconazole	0.5 mg/L
<i>A. stellatus</i>	amphotericin B	1 mg/L
	voriconazole	ATU
	isavuconazole	4 mg/L.
	posaconazole	1 mg/L
	itraconazole	ATU

Discussion

Antifungal drugs are employed in the treatment of fungal infections, however, antifungal resistant species such as *Aspergillus*, *Candida*, dermatophytes among others have been reported and, on the rise, (Arendrup, 2014). In our study, we found antifungal resistance to fluconazole and not to other azoles in South West Uganda, also corroborated by Ampaire *et al.* (2016) and Kainz *et al.* (2020). However, the majority of research conducted in Africa is concentrated on antibacterial resistance, with little to no research in the domain of antifungals and resistance in fungi so we cannot reach a definite conclusion on resistance prevalence. From an epidemiological point of view, less is known about the true prevalence of resistant *Aspergillus* spp infections compared to others, for instance, *Candida* infections. Probably due to the lack of national surveillance programmes in most African countries. For example, Uganda's Antimicrobial Resistance National Action Plan does not consider antifungals (UNAS, 2016).

In most African countries, there is widespread use of anti-fungal drugs in the poultry industry and information on the prevalence of antifungal resistance in isolates of animal and human origin is limited. In this study, seventeen different fungal species were isolated from the poultry feed samples and identified by ITS sequencing and MALDI-TOF. MALDI-TOF has become an effective way to detect fungal isolates but its success relies on the availability of exhaustive databases (Pierre Becker *et al.*, 2019) and expensive equipment not always available in African countries.

As observed by Kivumbi and Standley (2021), we also acknowledge that despite the known relevance of all three sectors in avoiding and moderating the spread of antimicrobial resistance (AMR), most studies on AMR in Uganda concentrated on human pathogens, overlooking animals and the environment however a one health approach is necessary. The results of these studies or evaluations of the larger determinants of AMR may have limited generalizability for the creation of new AMR surveillance strategies, they are nonetheless highly useful for directing decisions on appropriate treatment protocols for patients. An additional factor to the increase of AMR can be the desire to maximize economic profits which may lead veterinarians to aggressively encourage the use of antibiotics (Forman *et al.*, 2012). Thus, global food safety is a natural worry for human and animal health and the profitability of the market for other trading nations.

Fungal resistant infection such as in *A. fumigatus* is problematic as decreased survival of patients infected with these strains has been reported (Resendiz-Sharpe *et al.*, 2019), however, susceptibility testing is rarely undertaken for *Aspergillus* spp or fungi general in the clinical samples (Fructueux *et al.*, 2022). Furthermore, our knowledge of antifungal resistance in animal is much more limited. Therefore, our study sampled and analyzed the food fed to chickens to investigate fungal species which may be resistant to antifungals.

Resistance in *A. fumigatus* is problematic as patients infected with these strains will fail therapy but susceptibility is rarely undertaken for *Aspergillus* spp (Fructueux *et al.*, 2022). This necessitated the sampling of feed fed to chickens to analyse and investigate fungal species which may be resistant to antifungals.

Aspergillus tamari and *Aspergillus chevalieri* were used as fungal isolates against fluconazole showing no resistance to this antifungal. This antifungal drug is fungistatic rather than fungicidal, so treatment provides the opportunity for acquired resistance to develop in the presence of this antifungal drug. The development of fluconazole resistance in *Candida* spp. has been well characterized in *C. albicans* (Martínez *et al.*, 2002; Perea *et al.*, 2001) but not in filamentous fungi. There is still a lot we don't know about the resistance mechanisms to fluconazole outside of the conventionally accepted ones. The high level of resistance shown in many clinical isolates cannot often be fully explained by currently known molecular processes, emphasizing the significance of further research. High-level resistance is most frequently caused by multiple mechanisms that have been acquired over time over time. Pathogenic yeasts may become resistant to fungistatic azole medications like fluconazole over time, which would reduce the drug's effectiveness (Bhattacharya *et al.*, 2020). The most used azole for the prevention and treatment of *Candida* infections is fluconazole. But numerous *Candida* species have developed azole resistance, which is a new issue leading to therapeutic failures (Sanglard *et al.*, 2009).

Azoles and Amphotericin B are the recommended anti-*Aspergillus* medications for the treatment and prevention of *Aspergillus* infections (Whaley *et al.*, 2017). The most clinically significant species of *Aspergillus* that can cause *aspergillosis* is *A. fumigatus*, followed by *A. flavus*, *A. terreus*, *A. niger*, and other *Aspergillus* species (Alastruey-Izquierdo *et al.*, 2014). Given the important function of triazoles in the treatment of *aspergillosis*, azole resistance in *Aspergillus* spp. isolates continues to be a clinical problem. (Van Der Linden *et al.*, 2011; Ullmann *et al.*, 2018). Following the EUCAST-proposed breakpoints for some fungi, none of the tested fungal isolates: *Aspergillus flavus*, *A. ochraceus*, *A. niger*, *A. astellatus* and *Penicillium hetheringtonii* showed resistance/elevated MICs to the triazoles (isavuconazole, posaconazole, itraconazole) or amphotericin drugs. Azole resistance has been documented in several investigations with the reported fungi strains, particularly to ITZ, with high rates in European nations including the Netherlands and the United Kingdom, where azole resistance rates reached 38% (van Ingen *et al.*, 2015, Bueid *et al.*, 2010). This resistance rates have been attributed to long-term azole therapy in patients with chronic *aspergillosis*, in addition to the use and selection due to agricultural triazoles (Mortensen *et al.*, 2011, Snelders *et al.*, 2008).

Despite claims to the contrary, the extent and distribution of Azole Resistant *Aspergillus* species in Africa are still largely unknown (Negetu *et al.*, 2016; Yerbanga *et al.*, 2021; Resendiz-Sharpe *et al.*, 2021). Only a few studies from Nigeria, Kenya and Tanzania reported triazole resistance in *A. fumigatus* species (Fructueux, *et al.*, 2022). In Uganda, epidemiological data on the susceptibility of *Aspergillus* isolates recovered from the environment are quite uncommon because of the lack of regular susceptibility tests in most of the clinical laboratories. Despite the fact that our study was limited in its ability to examine a large number of fungi, it was still possible to draw the conclusion that a sizable percentage of the environmental strains of *Aspergillus* species were not azole-resistant.

Our study is the first to report the presence of fungus in poultry feed in Southwest Uganda. Compared to other Eastern African Countries, Uganda appears to have a substantially lower prevalence of ARAF than Tanzania (13.9 percent, and Kenya 27 percent. (Chowdhary *et al.*, 2016; Kemoi *et al.*, 2018). Both nations, have extensive azole-fungicide use in their intensive farming practices and engage in significant international trade, all of which raises the possibility of selecting resistant isolates and importing of Azole Resistant *Aspergillus flavus* (ARAF) from other nations. The absence of ARAF isolates in Kabale, Southwest Uganda might be due to factors such as the relative limited use of antifungals in the poultry sector. Yet, more research is needed in other districts in Uganda while extending the investigation to other animal feed.

Conclusions

This study described for the first time the presence of fungi isolates in poultry feed and those which are resistant to azole antifungal in poultry feed in South West Uganda. Given the prevalence of antimicrobial use in agricultural contexts, this outcome was unexpected. Our research supports the need for additional, in-depth environmental investigations that cover all of Uganda to verify the prevalence of azole-resistance and the necessity of azole-resistance surveillance in clinical settings.

Authors' Contributions

AO: Conceptualization, collection of samples, processing of samples, carrying out experiments, recording of results and manuscript writing; GV: methodology and review of manuscript; PVD: resources and review of manuscript; ARS: methodology and review of manuscript.

All authors read and approved the final manuscript

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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